The Nitroblue Tetrazolium Test in Black Patients

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SUMMARY

An outline of the development of the NBT test is given. The results of studies in a Black population are given and these conform to the pattern published elsewhere, except that a high degree of positivity was found in pulmonary tuberculosis. The value of the test is discussed, and it is concluded that, except in the rare condition of chronic granulomatous disease, the test is of very limited value in the diagnosis of bacterial infection. The test is positive in many situations of stress and may be mediated through a factor in the 'acute phase'.


Nitroblue tetrazolium is one of the tetrazole dyes, a group of colourless, soluble substances which, on reduction, yield water-insoluble pigments known as formazans. These dyes have been used in histochemistry to identify dehydrogenases and oxidases within cells. When phagocytosis occurs in granulocytes, an oxidase is activated in the phagocytic vacuole which forms substances with powerful bactericidal properties, hydrogen peroxide and superoxide. Holmes et al., investigating the increased susceptibility to infection of patients with chronic granulomatous disease, found that the granulocytes, while able to ingest bacteria normally, were unable to kill them, and this was subsequently shown by Baehner and Nathan to be the result of a leucocyte oxidase deficiency. Further studies showed that this deficiency of leucocyte oxidase could be demonstrated by the inability of granulocytes in chronic granulomatous disease to reduce nitroblue tetrazolium. It was then proposed that the qualitative BT test could be used to detect not only patients with chronic granulomatous disease, but also (the condition being X-linked recessive) female carriers, in whom one half of the granulocytes showed diminished NBT reduction.

Park et al. found that when blood from normal individuals was incubated in vitro with the dye, about 8.5% of the neutrophil granulocytes took up the dye and reduced it. Such granulocytes usually showed a coarse aggregation of the dye into a clump in the cytoplasm, and occasionally the reduced dye was in the form of scattered granules. These workers further noted that in bacterial infections the percentage of granulocytes reducing the dye was considerably increased and they suggested the use of the reaction for the identification of bacterial infection in febrile patients.

The reduction seen by Park et al. in such tests was regarded as 'spontaneous'. A 'stimulated' test was also devised, in which, on incubation with endotoxin in vitro, a considerably increased proportion of neutrophil granulocytes took up the dye; other workers have performed the test in the presence of bacteria or latex particles, to stimulate phagocytosis. In these modifications of the test, the percentage of neutrophils which can be 'stimulated' to take up and reduce the dye is measured to exclude false-negative results caused by faulty phagocytic mechanisms.

The aims of the present studies were to establish normal values for neutrophil NBT reduction for our Black population, to study the tests in various disease states in a Black hospital population, to examine the specificity of the test in this population and to consider the question of the value of the test in routine laboratory practice.

PATIENTS AND METHODS

Essentially, the test was performed as described by Park et al. Heparinised blood was used in the test. The concentration of heparin used was higher (300 U/ml blood) than that used by Park et al., and the incubation period was prolonged to 45 minutes at 37°C, followed by 15 minutes at room temperature. These modifications were necessary in order to obtain satisfactory results. In the presence of lower concentrations of heparin, a very low percentage of positivity was noted, while higher concentrations of heparin lead to excessive clumping of the leucocytes. Others have noted that heparin increases the sensitivity of the test. The heparinised blood was centrifuged in Wintrobe tubes and 0.1 ml of the leucocyte layer was mixed with 0.1 ml of a 0.1% solution of NBT in physiological saline, and was incubated as described. Slides of the incubated material were prepared, air-dried, and then stained with May-Grünwald-Giemsa stain. Counts of formazan-positive cells were made, by means of an oil-immersion objective (× 100) and were expressed as a percentage of the neutrophils and monocytes present. Only free leucocytes were enumerated; sometimes extensive aggregation of the leucocytes made enumeration difficult. Clumping of leucocytes can be prevented by the use of blood anticoagulated with Sequestrene, although this anticoagulant is said to reduce the sensitivity of the test.

Gordon et al. add Ficoll, the sucrose polymer, to restore sensitivity while retaining the advantages of Sequestrene as an anticoagulant. No attempt was made to stimulate NBT reduction by the addition of endotoxin, or by inducing phagocytic activity.

The 'normal' subjects studied were 17 Black blood donors, who were in good health at the time of testing. The patients studied were inpatients of the King Edward VIII Hospital in Durban. Three clinical groups were studied: a group of 25 patients with proved bacterial infections other than tuberculosis; a group of 25 patients with miscellaneous medical conditions but who were, as far as could be ascertained, without bacterial or other infection, and a group of 25 patients with pulmonary tuberculosis.
RESULTS

Normal Blacks

The values obtained in the normal group are shown in Table I, and are similar to those obtained by other authors in other race groups. It was important to establish this, since the leucocyte pattern is different in the Black, and leucopenia often exists with neutropenia in normal subjects. For this reason, the absolute numbers of NBT-positive neutrophils are shown in Table I; the values do not differ significantly from those given by Feigin et al. for their control group.

Black Hospital Patients

**Bacterial infections.** The group of patients with proved bacterial infections showed a significantly higher percentage of NBT-positive neutrophils than either the control group or the group of hospital patients with non-infective illnesses (see Table I).

**Non-infective disease.** The group of patients with non-infective illnesses did not differ significantly from the control group, but showed a slightly wider range of values.

**Pulmonary tuberculosis.** Pulmonary tuberculosis is a serious problem among the Blacks of Natal. The radiological diagnosis made on admission often does not differentiate between pulmonary tuberculosis and pyogenic pneumonia. It was of interest to determine whether the NBT test might assist in this differentiation, since the reported results of its use in tuberculosis (mostly very small series), suggested that the NBT test is negative. Our results contradict this (Table I). Some of our highest values were found in pulmonary tuberculosis. To account for this, the following possibilities were considered:

- The extensiveness of the tuberculous lesion. There is some evidence to suggest that miliary tuberculosis and tuberculous meningitis show increased NBT positivity, and this might be related to the widespread lesions in these conditions. In many of our patients who have pulmonary tuberculosis, the pulmonary disease is very extensive at first presentation.
- The tuberculous lesions are secondarily infected with pyogenic organisms. This is a well-recognized fact, and there is often considerable general improvement in patients with subsequently proved pulmonary tuberculosis who have been treated with antibiotics, which are ineffective against the tubercle bacillus.

There is increasing evidence that NBT positivity is more related to a 'first phase' reaction than to any specific response to bacterial infection. Many patients with pulmonary tuberculosis are extremely ill on admission, and this may account for the NBT positivity.

**Specificity of the NBT Test**

A disturbing feature of most series has been the considerable number of false-positives among controls and false-negatives among those with proved infections (Table II).

If absolute numbers of NBT-positive cells are considered instead of percentages, and a value of 800 positive cells/μl (= mean + 2SD) is taken as being the upper limit of normal, then the number of false-negatives among those with proved infections almost doubles itself (from 5/25 to 9/25). Feigin et al. cognisant of the frequent discrepancy between the percentage of positivity and absolute numbers of NBT-positive neutrophils, applied discriminant analysis to their data and produced a nomogram which categorised patients into one of four groups, depending on the relationship of these two measurements. The results of the NBT test were interpreted for each of our patients by the Feigin nomogram, and assigned to the appropriate Feigin group, viz. A, B, C or D. Only 2 patients with proved infection and 5 patients with pulmonary tuberculosis satisfied the criteria laid down for untreated bacterial infection. Most of our clinical material fell into the category B ('viral infection; partially but effectively treated bacterial infection; non-infectious fever'). It does not seem that the nomogram is helpful in spite of claims to the contrary.

**DISCUSSION**

The NBT test was originally introduced as a means of differentiating the neutrophilia of bacterial infections from those which arise from other causes. It was linked to the mechanism of phagocytosis by the findings of invariable

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>Range</th>
<th>SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td>17</td>
<td>280</td>
<td>78 - 1 105</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(8.3%)</td>
<td>(2 - 15%)</td>
<td>(0.9%)</td>
</tr>
<tr>
<td><strong>Patients — non-infectious disease</strong></td>
<td>28</td>
<td>353</td>
<td>0 - 1 216</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.7%)</td>
<td>(0 - 20%)</td>
<td>(1.0%)</td>
</tr>
<tr>
<td><strong>Patients — bacterial infections</strong></td>
<td>25</td>
<td>1 304</td>
<td>306 - 3 534</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(18.3%)</td>
<td>(4 - 36%)</td>
<td>(1.6%)</td>
</tr>
<tr>
<td><strong>Patients — pulmonary tuberculosis</strong></td>
<td>25</td>
<td>1 776</td>
<td>320 - 5 300</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20.5%)</td>
<td>(8 - 53%)</td>
<td>(2.2%)</td>
</tr>
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</table>

* Standard error of the mean.
Comparison of controls and non-infectious disease P>0.1.
Comparison of controls and bacterial infections P<0.001.
Comparison of controls and pulmonary tuberculosis P<0.001.
Thus, while some authors have recommended NBT positivity as a method for the identification of the rare, hereditary conditions associated with chronic granulomatous disease, and its carrier state, it is becoming clear that preoccupation with bacterial infection as a cause of NBT positivity may be misleading. There is considerable evidence that NBT positivity may be merely an expression of an effect of an 'acute phase' reaction on the neutrophils. Thus, Lauter et al.22 and Shafar et al.23 have found that NBT positivity is present during the period immediately after a myocardial infarction. Segal et al.24 in a re-appraisal of the NBT test, conclude that the test reflects the existence of a situation of stress, rather than defines its cause.

A completely standardised procedure is not yet available for this test, and this must be remembered when results from different authors are compared. It probably accounts for the widely divergent views expressed in the literature regarding the practical value of the test.21,16,17,19 It is our view that the test is of very limited value in the routine investigation of a febrile patient, but that it is undoubtedly of value in the identification of the rare, hereditary condition of chronic granulomatous disease, and its carrier state.

NBT negativity in neutrophils in chronic granulomatous disease; the hypothesis was that when neutrophils were involved in phagocytosis in vivo by a bacterial infection, metabolic changes, which could be detected by increased reduction of the NBT dye, occurred.

Since these views were advanced, numerous anomalous findings in a variety of conditions have cast doubt upon them. It was then suggested20 that the infection must be systemic (i.e. not local), and that the phagocytic system must be operating normally. However, in a prospective study of patients with positive blood cultures, only 50% showed a positive NBT test,20 while Matula and Paterson15 reported positive NBT tests in almost 90% of instances of infection without bacteremia. Many conditions in which false-negative NBT tests are found have been described.25,26 Park and Good27 suggested that, in such cases, the defect might lie in the phagocytic function of the neutrophil; to overcome this they suggested that a 'stimulated' test with endotoxin be performed. However, there is little evidence that phagocytosis is involved in the NBT test. How the dye penetrates the cell is not understood; according to Pearce,28 the normal cell membrane is impermeable to the dye. Other modes of entry that have been postulated are the uptake of dye by platelets and phagocytosis of the platelets by neutrophils, or the deposition of reduced dye in granular form on the external surface of the neutrophil and subsequent phagocytosis.29 It does seem clear, however, that any insult to the neutrophil (physical, chemical, or enzymatic) will result in a high positivity for NBT staining of the cells.22 Thus, while some authors have recommended techniques which will result in such damage as being useful modifications of the NBT test,24,25 others28 have advocated a technique involving minimal 'stimulation' (?) trauma) of the neutrophils used in the test.

### TABLE II. SPECIFICITY OF THE NBT TEST

<table>
<thead>
<tr>
<th></th>
<th>False positives</th>
<th>False negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>On percentages*</td>
<td>On absolute number</td>
</tr>
<tr>
<td>Controls</td>
<td>3/17 (18%)</td>
<td>1/17 (6%)</td>
</tr>
<tr>
<td>Non-infectious disease</td>
<td>4/25 (16%)</td>
<td>3/25 (12%)</td>
</tr>
<tr>
<td>Bacterial infections</td>
<td></td>
<td>5/25 (20%)</td>
</tr>
<tr>
<td>Pulmonary tuberculosis</td>
<td></td>
<td>9/25 (36%)</td>
</tr>
</tbody>
</table>

* Upper limit of normal (mean + 2 SD) taken as being 800/μl (mean + 2 SD).

### TABLE III. PREDICTION BY FEIGIN NOMOGRAM

<table>
<thead>
<tr>
<th></th>
<th>Prediction by Feigin nomogram</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Controls</td>
<td>16</td>
</tr>
<tr>
<td>Non-infectious disease</td>
<td>22</td>
</tr>
<tr>
<td>Bacterial infections</td>
<td>6</td>
</tr>
<tr>
<td>Pulmonary tuberculosis</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
</tr>
</tbody>
</table>

A = controls; B = viral infection, partly treated bacterial infection and non-infectious febrile illnesses; C = untreated bacterial infection; D = ineffectively treated bacterial infection.

26. Greig, H. B. W.: To be Published.